THE EMBO JOURNAL

Manuscript EMBO-2015-92244

Presynaptic inhibition upon CB1 or mGlu2/3 receptor activation requires ERK/MAPK phosphorylation of Munc18-1

Sabine K Schmitz, Cillian King, Christian Kortleven, Vincent Huson, Tim Kroon, Josta T Kevenaar, Desiree Schut, Ingrid Saarloos, Joost P Hoetjes, Heidi de Wit, Oliver Stiedl, Sabine Spijker, Ka Wan Li, Huibert D Mansvelder, August B Smit, Lennart Niels Cornelisse, Matthijs Verhage, Ruud F Toonen

Corresponding author: Matthijs Verhage, Vrije Universiteit Amsterdam

Review timeline:

Submission date:	08 June 2015
Editorial Decision:	01 July 2015
Revision received:	18 January 2016
Editorial Decision:	16 February 2016
Revision received:	28 February 2016
Accepted:	02 March 2016

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 01 July 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, both referees find the analysis interesting. However they also find that further analysis is needed to validate the key conclusions in order to consider publication here. The referees raise constructive comments and I expect that you should be able to resolve them in a good way.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript. As you know it is EMBO Journal policy to allow a single round of major revision only and that it is therefore important to resolve them at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this Ms Verhage, Toonen and colleagues unravel an important component of an ERK-dependent pathway of presynaptic short-term inhibition that can be driven by postsynaptically released endocannabinoids via presynaptic CB1 receptors or mGluR dependent pathways of homeostatic plasticity. Specfically, they convincingly demonstrate that activated ERK phosphorylates the essential presynaptic release factor Munc18-1 at multiple sites, most importantly S241A in vitro and in living animals in vivo as beautifully demonstrated by a fear conditioning paradigm. Munc18-1 phosphorylation by ERK1/2 is regulated by neuronal network activity as well as external cues, i.e. BDNF stimulation or CB1 receptor activation. Mechanistically, it is shown using viral rescue experiments in autaptic cultures from Munc18-1 knockout mice that expression of a non-ERK phosporylatable mutant version of Munc18-1 (S241A) increases both evoked as well as spontaneous presynaptic neurotransmitter release and elevated the size of the readily releasable pool (RRP). Conversely, hyperphosphorylation of Munc18-1 mimicked by expression of S241D mutant Munc18-1 is associated with reduced synapse number, reduced evoked and spontaneous neurotransmission, and elevated SV numbers resulting from a reduced release probability. This impaired basal neurotransmission caused by Munc18-1 S241D originates from facilitated degaradtion of the mutant Munc18-1 protein by the ubiquitin-proteasome pathway. Finally, the authors demonstrate that ERK-mediated phsophorylation of Munc18-1 prevents endocannabinoid- and mGluR induced presynaptic depression in hippocampal autaptic neurons in culture.
Overall, this beautiful new study convincingly demonstrates a novel role for ERK-mediated phosphorylation of Munc18-1 as a key mechanism underlying presynaptic short-term inhibition in vitro and in vivo. These findings are of high interest to the neuroscience community as well as to cell biologists interested in neurotransmisson.

I have only a few minor suggestions for improvement of the Ms.
1. In Fig. 5H it appears that S241A is expressed at elevated levels compared to the WT protein, while both proteins seem to be present at similar levels when assayed by immunofluorescence in panels B and C. The authors may want to resolve this discrepancy.

2. The discussion seems a bit superficial. I suggest to discuss in a some more detail how the new results fit with previously described mechanisms of presynaptic inhibition via CB1, e.g. the pssible involvement of calcium channels at inhibitory synapses.

3. The resolution of panel C in Fig. 4 seems low and would need improvement to reach publication quality.

Referee #2:

Schmitz et al report that presynaptic inhibition mediated by cannabinoid and metabotropic receptors (CB1R and mGluR2/3) require ERK-dependent phosphorylation of Munc18-1. The two main conclusions are: a) Munc18-1 is a presynaptic ERK substrate; b) ERK-dependent Munc18-1 phosphorylation reduces synaptic transmission and could be involved in endocannabinoid and mGluR2/3-mediated suppression of glutamate release. If true, the findings seem to challenge current views on the mechanism by which these receptors transiently suppress transmitter release. The study presents a number of experimental approaches that do not necessarily talk to each other. While most of the experiments are well designed, a number of the authors' claims are not fully supported by the data. Moreover, the study heavily relies on the overexpression of two Munc18-1 mutants in autaptic neurons. Given the potential impact of the authors' claims, the main conclusions of this study should be validated using alternative approaches and by testing a few predictions.

MAJOR CONCERNS

1. Several studies have demonstrated that a reduction in presynaptic calcium influx is responsible for the fast, transient suppression of transmitter release upon activation of CB1Rs or mGluRs. In contrast, the authors suggest that ERK-dependent phosphorylation of Munc18-1 is a major mechanism in this transient suppression. It is possible that the authors' findings could be due to the

rather non-physiological approach used in this study -e.g. overexpression of mutant forms of Munc18-1 in autaptic neurons. If both reduction in calcium influx and Munc18-1 phosphorylation coexisted, the authors should determine their relative contribution under more physiological conditions. At least they should test whether PD98059 and some other ERK-pathway inhibitor blocks WIN-mediated suppression of transmission and Depolarization-induced Suppression of Excitation (DSE) in both autaptic neurons and acute brain slices.

2. The authors claim that Munc18-1 gets phosphorylated by activation of presynaptic receptors that are implicated in negative feedback signaling. However, the mechanism by which BDNF and LY379268 promote Munc18-1 phosphorylation could be indirect and not necessarily presynaptic. Moreover, they also claim that ERK-dependent Munc18-1 phosphorylation plays a key role in multiple retrograde signaling pathways. However, not a single manipulation directly tests this possibility, and only pharmacological tools were used. As indicated above, they should examine the role of phosphorylated Munc18-1 on endocannabinoid signaling (e.g. by testing DSE). As for mGluR2/3 activation, it is important to know whether LY379268 reduces transmitter release in naïve autapses (PPF, failure rate, mEPSC).

3. It appears that ERK increases transmitter release by phosphorylating synapsin but decreases transmitter release by phosphorylating Munc18-1. How does it work? What is the functional consequence of phosphorylated Munc-18 and phosphorylated syntaxin on neuron output? Do these actions occur in the same synapses? The authors claim different time domains but the evidence in support of this possibility does not seem to be that strong. This issue should be thoroughly discussed.

4. Does the WIN-induced suppression wash out? Show recovery post washout.

5. Consistent with a reduction in both evoked EPSC amplitude and mEPSC frequency, PPR was increased by expressing the S241D mutant in autaptic neurons. In contrast, PPR was unchanged by the S241A mutant, which clearly increased evoked transmission, mEPSC activity and RRP size. Do the authors have an explanation for this dissociated phenotype?

OTHER POINTS

Abstract, first sentence: presynaptic CB1Rs regulate synaptic strength not only by *transiently" inhibiting secretion. CB1Rs also mediate long-term suppression of neurotransmitter release (even in autaptic neurons; see Kellog et al, J. Neurophysiol, 2009). Also, while the effects of both CB1R and mGluR2/3 were analyzed in this study, only CB1Rs are mentioned in the abstract.

Page 8, ERK auto-phosphorylation, provide some quantification.

Page 11, Synaptic Munc-18 levels, describe how synaptic v. somatic Munc-18 was quantified. Not listed in the text or Methods.

Page 14, line 2, "...to produce presynaptic inhibition (Derkinderen et al, 2003; Kellogg et al, 2009)". Wrong citations, revise.

Page 14, lines 4-6: indicate in the text what preparation (cells/tissue) was used in these experiments.

Page 17, lines 5-7: provide some references.

The discussion is extremely short and should be expanded.

The title is too open ended (CB1R or mGluR2/3 should be mentioned).

1st Revision - authors' response 18 January 2016

Reply to Referee #1:

We thank the reviewer for his/her positive evaluation of our manuscript (*"beautiful new study"*). This reviewer concludes that the manuscript will be "*of high interest to the neuroscience community as well as to cell biologists*". This reviewer raises 3 minor issues:

1: *In Fig. 5H it appears that S241A is expressed at elevated levels compared to the WT protein, while both proteins seem to be present at similar levels when assayed by immunofluorescence in panels B and C. The authors may want to resolve this discrepancy.*
Reply: The reviewer is correct. In Figure 5H we perform measurements of WT and S241A levels in cultures with 3-times more cells/well than in Figure 5B-C, which increases spontaneous activity and activates ERK. In these cultures S241A levels are increased compared to WT, as predicted. These high-density cultures were also used for analysis in Figure 5E (yielding similar results). We have added a better description to the result section at page 13 lines 5-7

2: *The discussion seems a bit superficial. I suggest to discuss in a some more detail how the new results fit with previously described mechanisms of presynaptic inhibition via CB1, e.g. the possible involvement of calcium channels at inhibitory synapses.*
Reply: we agree with the reviewer and we have now thoroughly revised the discussion section to discuss our findings in the light of previously described mechanisms (including involvement of calcium channels).

3: *The resolution of panel C in Fig. 4 seems low and would need improvement to reach publication quality.*
Reply: this was likely caused by the pdf conversion in the original submission, panel 4C in the final figure is now of high resolution.

Reply to Referee #2:

We thank the reviewer for his/her thorough and balanced review of our manuscript. The reviewer argues that given the potential impact of our findings, validation via alternative approaches is required. This reviewer raises 5 major issues:

1. *Several studies have demonstrated that a reduction in presynaptic calcium influx is responsible for the fast, transient suppression of transmitter release upon activation of CB1Rs or mGluRs. In contrast, the authors suggest that ERK-dependent phosphorylation of Munc18-1 is a major mechanism in this transient suppression. It is possible that the authors' findings could be due to the rather non-physiological approach used in this study -e.g. overexpression of mutant forms of Munc18-1 in autaptic neurons. If both reduction in calcium influx and Munc18-1 phosphorylation coexisted, the authors should determine their relative contribution under more physiological conditions. At least they should test whether PD98059 and some other ERK-pathway inhibitor blocks WIN-mediated suppression of transmission and Depolarization-induced Suppression of Excitation (DSE) in both autaptic neurons and acute brain slices.*
Reply: We agree with the reviewer that some of the conditions used in our experiments are "rather non-physiological". This is of course common practice in many published experiments on the mechanisms of synaptic plasticity. As requested, we tested the effect of ERK inhibitors on DSE in hippocampal slices (new Figure 7A-C) and autaptic neurons (new Figure S7B-C) and on WIN-mediated suppression in hippocampal slices (new Figure 7D-F) and autaptic neurons (new Figure S7D-F). We wish to point out that these conditions, ERK-pathway inhibition and DSE induction, are also "rather non-physiological".
In hippocampal slices, WIN-mediated depression and DSE were reduced by >60% by ERK-pathway inhibition. This confirms that the ERK/Munc18 pathway is indeed a major pathway in CB1R-mediated depression of synaptic transmission. It also leaves room for other pathways like reduction of presynaptic calcium influx to contribute to CB1R-mediated depression. We address this now more explicitly in the Discussion section, also the fact that our data do not challenge the CB1-dependent calcium channel inhibition. The new mechanism most likely co-exists with channel inhibition. In autaptic neurons, we found it was impossible to obtain good inhibition of the ERK-pathway using PD98059 and U0126. These blockers did not reduce phospho-ERK levels in all neurons, as we found out in control experiments, directly assessing ERK-induced phosphorylation of its substrates (New Figure S7G, H) Although inhibition was incomplete, it significantly reduced the amplitude and duration of DSE and blocked WIN-mediated suppression in a subset of neurons

(20%) but not in all. Our previous data provided some clues on the reasons why inhibition was incomplete in autaptic neurons. ERK itself appears to remain in a phosphorylated state much longer than in hippocampal slices (Fig, 3C). We have added a short discussion on this possibility to the legend of Fig S7, but since the effects of ERK-inhibition were robust and conclusive in slices, we decided not to pursue ERK-inhibition in autaptic neurons further.

2*: The authors claim that Munc18-1 gets phosphorylated by activation of presynaptic receptors that are implicated in negative feedback signaling. However, the mechanism by which BDNF and LY379268 promote Munc18-1 phosphorylation could be indirect and not necessarily presynaptic. Moreover, they also claim that ERK-dependent Munc18-1 phosphorylation plays a key role in multiple retrograde signaling pathways. However, not a single manipulation directly tests this possibility, and only pharmacological tools were used. As indicated above, they should examine the role of phosphorylated Munc18-1 on endocannabinoid signaling (e.g. by testing DSE). As for mGluR2/3 activation, it is important to know whether LY379268 reduces transmitter release in naïve autapses (PPF, failure rate, mEPSC).*
Reply: We agree with the reviewer that BDNF and LY379268-induced phosphorylation of Munc18-1 may be indirect and not presynaptic. These compounds were used to show that several pathways that lead to ERK activation result in phosphorylation of Munc18-1. Two of these compounds, LY379268 and WIN, which activate presynaptic receptors and lead to ERK activation resulted in a reduction of synaptic transmission that depended on Munc18 phosphorylation. We have now assessed the contribution of this ERK-pathway in DSE and WIN-mediated suppression of release in slices and autapses to show that this pathway significantly contributes to CB1R-mediated suppression of release. See also our answer to Q1 above.
The group of Richard Miller has studied the effect of mGluR2/3 activation in autaptic cultures (Bushell et al., 1999). They find a similar reduction of EPSC amplitude and mEPSC frequency in wild type autapses upon mGluR2/3 activation as we find in munc18-1 null mutant autapses rescued with $M18_{WT}$. Hence, the effect of both WIN and LY379268 application on synaptic transmission is similar in WT autapses and munc18-1 null mutant autapses rescued with $M18_{WT}$. We added this to the result section on page 16.

3. It appears that ERK increases transmitter release by phosphorylating synapsin but decreases transmitter release by phosphorylating Munc18-1. How does it work? What is the functional consequence of phosphorylated Munc-18 and phosphorylated synapsin on neuron output? Do these actions occur in the same synapses? The authors claim different time domains but the evidence in support of this possibility does not seem to be that strong. This issue should be thoroughly discussed.
Reply: We agree that this issue deserves more discussion. We have now devoted a paragraph in the Discussion to this topic (p 19). We have emphasized that our data confirm ERK-dependent synapsin phosphorylation, (and now show that it occurs during fear conditioning); that phosphorylation of synapsin and Munc18-1 probably occurs in the same synapses, but that the evidence for the relevance of ERK-dependent synapsin phosphorylation is based on PD98059 application (Jovanovic et al 2000), i.e. manipulations that block positive as well as negative actions of ERK-substrates. To our knowledge, the critical experiment, to express a phosphorylation-deficient synapsin mutant on a (triple) null background, like we present for Munc18-1, has not been performed. In fact, EPSC amplitudes are not affected in the synapsin triple knock-out, (Gitler et al., 2004). We agree with the reviewer that the evidence in support of different time domains is not strong. We have removed this idea in the revised Discussion.

4. Does the WIN-induced suppression wash out? Show recovery post washout.
Reply: In figure 7 we apply WIN for only 30 sec and observe a strong suppression in EPSC amplitude that lasts at least 4 minutes (Figure 6B and E). WIN-induced suppression is reversible in both autaptic neurons and in slices but recovery is relatively slow requiring on average 30 minutes after WIN application. We added a supplemental figure showing WIN washout in slices and autapses (new figure S6).

5. Consistent with a reduction in both evoked EPSC amplitude and mEPSC frequency, PPR was increased by expressing the S241D mutant in autaptic neurons. In contrast, PPR was unchanged by the S241A mutant, which clearly increased evoked transmission, mEPSC activity and RRP size. Do the authors have an explanation for this dissociated phenotype?

Reply: The PP facilitation phenotype of the S214D mutant closely resembles the CAPS DKO (Jokusch et al., 2007) in which both RRP and Pvr are reduced and PPR is increased. However, PPR is not dependent on RRP size and a larger RRP does not necessarily lead to a reduced PPR. This has been shown by (Matveev and Wang, 2000, and Sullivan 2007) who conclude that the release of a single vesicle will not deplete the RRP enough to cause paired-pulse depression. We added this to the result section at p 12. This can also be proven mathematically:

$$Fused_1 = RRP_1 P_{vr1}$$

$$Fused_2 = RRP_2 P_{vr2}$$

$$RRP_2 = RRP_1 - Fused_1 = RRP_1\left(1 - P_{vr1}\right)$$

with $Fused_1$ and $Fused_2$ the number of fused vesicles after the first and second pulse, respectively, RRP_1 and RRP_2 the readily releasable pool at the time of first and second pulse, respectively, and Pvr_1 and Pvr_2 the vesicular release probability at the time of first and second pulse, respectively. Then it follows that PPR, defined as $Fused_2/Fused_1$ is independent of the initial RRP size:

$$\frac{Fused_2}{Fused_1} = \frac{RRP_2 P_{vr2}}{RRP_1 P_{vr1}} = \frac{RRP_1\left(1 - P_{vr1}\right) P_{vr2}}{RRP_1 P_{vr1}} = \frac{\left(1 - P_{vr1}\right) P_{vr2}}{P_{vr1}}$$

OTHER POINTS

*Abstract, first sentence: presynaptic CB1Rs regulate synaptic strength not only by *transiently" inhibiting secretion. CB1Rs also mediate long-term suppression of neurotransmitter release (even in autaptic neurons; see Kellog et al, J. Neurophysiol, 2009). Also, while the effects of both CB1R and mGluR2/3 were analyzed in this study, only CB1Rs are mentioned in the abstract.*
Reply: we fully agree with the reviewer and changed the abstract and removed “transiently” and added a statement on mGluR2/3. We thank the reviewer for pointing this out.

Page 8, ERK auto-phosphorylation,provide some quantification.
Reply: we added quantification of the ERK auto-phosphorylation to figure 2F.

Page 11, Synaptic Munc-18 levels, describe how synaptic v. somatic Munc-18 was quantified. Not listed in the text or Methods.
Reply: we are sorry for this omission and added description to the methods section at p27.

Page 14, line 2, "...to produce presynaptic inhibition (Derkinderen et al, 2003; Kellogg et al, 2009)". Wrong citations, revise.
Reply: we thank the reviewer for pointing this out and changed the citations.

Page 14, lines 4-6: indicate in the text what preparation (cells/tissue) was used in these experiments.
Reply: we have added this to the main text at p14.

Page 17, lines 5-7: provide some references. Reply: we added references for these statements. The discussion is extremely short and should be expanded.
Reply: we agree with the reviewer and we have now thoroughly revised the discussion to discuss our findings in light of previously described mechanisms.

The title is too open ended (CB1R or mGluR2/3 should be mentioned).
Reply: we agree and changed the title to: “Presynaptic inhibition upon CB1 and mGlur2/3 receptor activation requires ERK/MAPK phosphorylation of Munc18-1”

References:
Bushell TJ, Lee CC, Shigemoto R, Miller RJ. (1999) Modulation of synaptic transmission and differential localisation of mGlus in cultured hippocampal autapses. Neuropharmacology. 38(10):1553-67.

Gitler D, Takagishi Y, Feng J, Ren Y, Rodriguiz RM, Wetsel WC, Greengard P, Augustine GJ. (2004) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. J Neurosci; 24(50):11368-80.
Jockusch WJ, Speidel D, Sigler A, Sørensen JB, Varoqueaux F, Rhee JS, Brose N. (2007) CAPS-1 and CAPS-2 are essential synaptic vesicle priming proteins. Cell;131(4):796-808.
Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat Neurosci 3: 323-329
Kellogg R, Mackie K, Straiker A (2009) Cannabinoid CB1 receptor-dependent long-term depression in autaptic excitatory neurons. J Neurophysiol 102: 1160-1171
Sullivan JM1 (2007) A simple depletion model of the readily releasable pool of synaptic vesicles cannot account for paired-pulse depression. J Neurophysiol; 97(1):948-50.
Matveev V, Wang XJ. (2000) Implications of all-or-none synaptic transmission and short-term depression beyond vesicle depletion: a computational study. J Neurosci; 20(4):1575-88.

2nd Editorial Decision 16 February 2016

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee 2 and the comments are provided below. As you can see, the referee appreciates the introduced changes and supports publication here. There are just a few minor issues to sort out before formal acceptance here.

REFEREE REPORT

Referee #2:

The authors have properly addressed most of my concerns by adding new results and significantly expanding Discussion. I only have a few minor comments that the authors may want to address.

- In page 16, the authors conclude: "Hence, multiple retrograde signaling pathways activate ERK-dependent Munc18 phosphorylation, which reduces synaptic output." What are the "multiple retrograde signaling pathways" the authors refer to? This conclusion is rather misleading since their study analyzes only one retrograde signaling pathway.

- The authors indicate that PD98059 and U0126 have no effect on glutamatergic autaptic transmission (Fig. S7A, note that figure legend and figure panel do not match). They should provide some information about EPSC time course. In addition, they should test whether EPI affect evoked synaptic transmission in acute slices by monitoring fEPSPs (e.g 20-30 min baseline, and at least one hour EPI bath application).

- Page 17, line 4, "Xu et 2010": DSE in CA1 pyramidal neurons was firstly shown by Ohno-Shosaku et al J. Neurosci 2002.

2nd Revision - authors' response 28 February 2016

Referee #2:

- In page 16, the authors conclude: "Hence, multiple retrograde signaling pathways activate ERK-dependent Munc18 phosphorylation, which reduces synaptic output." What are the "multiple retrograde signaling pathways" the authors refer to? This conclusion is rather misleading since their study analyzes only one retrograde signaling pathway.
Reply: the "multiple retrograde signaling pathways" referred to the multiple receptor pathways. We agree that our phrasing might be unclear and changed the sentence on p16 to “activation of multiple receptor systems leads to ERK-dependent Munc18 phosphorylation, which reduces synaptic output”.

- *The authors indicate that PD98059 and U0126 have no effect on glutamatergic autaptic transmission (Fig. S7A, note that figure legend and figure panel do not match). They should provide some information about EPSC time course. In addition, they should test whether EPI affect evoked synaptic transmission in acute slices by monitoring fEPSPs (e.g 20-30 min baseline, and at least one hour EPI bath application).*
Reply: To show that PD98059 and U0126 have no effect on glutamatergic autaptic transmission we have added example EPSC traces prior to and after EPI treatment to Figure EV5C and average EPSC rise and decay times prior to WIN55,212-2 application to Figure EV5F. In addition, we have added average EPSC amplitudes from control and EPI-treated neurons during baseline recordings prior to DSE and WIN application in acute slices as new Figure 7A and 7E, as requested by the reviewer. Together, these additional experiments show that PD98059 and U0126 have no effect on glutamatergic transmission in autapses and hippocampal slices.

- *Page 17, line 4, "Xu et 2010": DSE in CA1 pyramidal neurons was firstly shown by Ohno-Shosaku et al J. Neurosci 2002.*
Reply: we have added this reference to the text and apologize for the omission.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: RFG Toonen
Journal Submitted to: EMBOJ
Manuscript Number: 2015-92244

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For figure 2, based on publised data we expected a large effect size upon footshock (0.7-0.8) based on this assumption the group size (5 mice/group) resulted in a calculated a prior power of 0.8
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size were chosen to ensure adequate power with the expected effect sizes (see above). P33
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The experimentator was blind to genotype and/or compound in all experiments in the manuscript.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were dived randomly in 3 groups by animal caretakers that did not excecute the experiment. P30
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	The experimentator was blind to genotype and/or compound in all experiments in the manuscript.P33
4.b. For animal studies, include a statement about blinding even if no blinding was done	The experimentator was blind to genotype. After foot shock experiment hippocampi were extracted, anonymized and transferred other experimentator for protein analysis. P33Anonymization code was broken after full analysis of protein expression profiles (Figure 2).
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, data samples were tested for normal distribution with the Kolmogorov and Smirnov test
Is there an estimate of variation within each group of data?	Yes, groups were tested for heterogeneity of variance with the method of Bartlett. For multiple group comparisons, one-way ANOVA was used if allowed otherwise the non-parametric Kruskal-Wallis test was used.
Is the variance similar between the groups that are being statistically compared?	Not always, in case of unequal variance Welch correction was used or KW test for >2 groups.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	p26
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	p26

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male 10-week old C57BL/6J mice (Charles River) were used for behavioral analysis. Munc18-1 null mutant mice were generated as described (Verhage et al, 2000). Newborn P0-P1 Wistar rats (Harlan) were used for rat neuronal cultures and glia preparations P26, 32
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animals were housed and bred according to institutional and Dutch governmental guidelines. All animals were handled according to approved VU University Animal Ethics and Welfare Committee protocols (DEC-FGA-13-03 and DEC-FGA-14-01). P26
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We fully complied to the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	OK
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Yes
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No